



Sympathetic stimulation of thiazide-sensitive sodium chloride cotransport in the generation of salt-sensitive hypertension

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DOI: <https://doi.org/10.1161/HYPERTENSIONAHA.114.03335>

Posted at the Zurich Open Repository and Archive, University of Zurich

ZORA URL: <https://doi.org/10.5167/uzh-100805>

Journal Article

Published Version

Originally published at:

Terker, Andrew S; Yang, Chao-Ling; McCormick, James A; Meermeier, Nicholas P; Rogers, Shaunessy L; Grossmann, Solveig; Trompf, Katja; Delpire, Eric; Loffing, Johannes; Ellison, David H (2014). Sympathetic stimulation of thiazide-sensitive sodium chloride cotransport in the generation of salt-sensitive hypertension. *Hypertension*, 64(1):178-184.

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Hypertension. 2014;64:178-184; originally published online May 5, 2014;

doi: 10.1161/HYPERTENSIONAHA.114.03335

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

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Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:

<http://hyper.ahajournals.org/content/64/1/178>

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Key Words: diuretics ■ hypertension ■ ion transport ■ sodium-potassium-chloride symporters ■ sympathetic nervous system

Excessive renal sympathetic nerve activity has been proposed to be a final common pathway for the genesis of hypertension,¹ the largest global contributor to premature death.² The sympathetic nervous system seems to be especially important in individuals with an exaggerated response to dietary NaCl (salt-sensitive hypertensives), whether this results from genetic³ or acquired factors.^{4,5} Norepinephrine infusion in animals reduces urinary sodium excretion and increases blood pressure,^{6–8} but these effects may not reflect direct stimulation of salt reabsorption by renal tubules. Renal nerves also stimulate renin secretion, which increases sodium reabsorption and raises blood pressure via the actions of angiotensin II and aldosterone. Additional effects of the sympathetic nervous system on the renal vasculature may also contribute by altering renal hemodynamics. Where direct stimulation of Na⁺ reabsorption

by tubules has been invoked, it is generally accepted that α -adrenergic receptors mediate these effects.¹

More recently, Mu et al⁸ reported that chronic adrenergic activation, by norepinephrine or isoproterenol, causes salt-sensitive hypertension by activating the thiazide-sensitive NaCl cotransporter (NCC) in the kidney; they postulated that this effect was mediated by glucocorticoid-dependent effects on WNK kinases, specifically serine/threonine-protein WNK4. WNK effects are mediated primarily by the activation of 2 kinases, STE20/SPS1-related proline- and alanine-rich kinase (SPAK; STK39) and oxidative stress–response kinase 1 (OxSR1; *OxSR1*, often termed OSR1; because OSR1 is the official gene symbol for odd-skipped related 1, we have used the term OxSR1 for clarity). These findings have been controversial, because some aspects were not corroborated by

Received February 12, 2014; first decision February 27, 2014; revision accepted April 8, 2014.

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The online-only Data Supplement is available with this article at <http://hyper.ahajournals.org/lookup/suppl/doi:10.1161/HYPERTENSIONAHA.114.03335/-DC1>.

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Hypertension is available at <http://hyper.ahajournals.org>

DOI: 10.1161/HYPERTENSIONAHA.114.03335

another group,⁷ and other mediators, such as angiotensin II, were felt likely to have contributed.⁹ Interestingly, however, a little-cited study published >30 years ago, using micropuncture techniques, reported that acute isoproterenol infusion stimulated salt transport along the distal convoluted tubule (DCT).¹⁰ Additionally, the work of Morel¹¹ on adenylyl cyclase activities in microdissected nephron segments showed that the early distal tubule (corresponding to the DCT) of the rat is isoproterenol-responsive. If confirmed, these results would provide novel mechanistic insights linking salt and the adrenergic nervous system that are likely to have therapeutic implications.

During the past 10 years, interest in renal sympathetic control has surged, because technical advances have made renal denervation popular for treating resistant hypertension.¹² Substantial effects of renal nerve ablation on arterial pressure have been observed in many studies, using a variety of experimental models¹; early success in clinical trials¹³ led to wider adoption of the technique in Europe and high anticipation for Food and Drug Administration approval in the United States. The recent announcement that the large randomized renal denervation trial, SYMPPLICITY HTN-3,¹⁴ did not reach its primary efficacy end point, when compared with sham treatment, was disappointing, but it suggests that maximal medical treatment of humans, as achieved in this trial, may interrupt many of the pathways activated by renal nerves.

Although excessive activity of NCC is known to cause hypertension in patients with the Mendelian disease, familial hyperkalemic hypertension (pseudohypoaldosteronism type 2),^{15–17} many investigators suggest that such monogenic syndromes are not relevant for typical hypertension.¹⁸ In common forms of hypertension, however, a role for NCC is suggested by the substantial clinical efficacy of NCC inhibitors, thiazide diuretics, to treat hypertension. In fact, it is often forgotten that these drugs seem to be effective only when hypertension is present; they have little or no effect on arterial pressure in normotensive individuals, but reduce it substantially in hypertensive patients.^{19–21} Therefore, it would be appealing to find a link between sympathetic overactivity and activated NCC. Here, we confirmed that NCC is activated in salt-sensitive hypertension resulting from adrenergic stimulation. Because sympathetic nerve effects are typically rapid, we developed an acute model of adrenergic stimulation, permitting us to study signaling mechanisms *in vivo* in the absence of chronic compensation. This allowed us to confirm the involvement of β -adrenergic receptors and establish that activation is mediated by an atypical signaling pathway in the DCT.

Methods

See Methods in the online-only Data Supplement.

Results

To confirm the effects of chronic adrenergic stimulation on blood pressure, we infused mice with norepinephrine via osmotic minipump for 2 weeks according to the protocol previously described.⁸ Norepinephrine infusion increased systolic blood pressure slightly while mice consumed a normal salt diet (0.49% NaCl); when the mice consumed a high-salt diet (8% NaCl) with continued norepinephrine infusion, however, systolic pressure increased substantially (Figure 1A). In

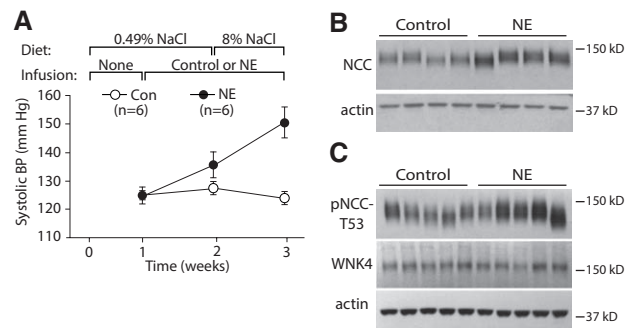


Figure 1. Confirmation that chronic norepinephrine (NE) infusion causes salt-sensitive hypertension and increases NaCl cotransporter (NCC) and phosphorylated NCC (pNCC). **A**, NE infusion caused salt-induced hypertension. During week 1, all animals were maintained on a normal salt diet without infusion. NE or control infusion (Con) was started during week 2, whereas normal salt diet was continued. During week 3, both groups were switched to a high-salt diet; $n=6$ per group. Differences were determined by 2-way ANOVA with repeated measures, where P values are 0.0018 for time, 0.0073 for treatment (NE vs Con), and 0.0119 for interaction. **B**, NE infusion caused an increase in NCC abundance compared with control mice; $n=4$ per group; $P<0.05$ by unpaired t test. **C**, NE infusion caused an increase in pNCC-T53 abundance compared with control mice ($P<0.05$ by unpaired t test); however, WNK4 abundance remained unchanged; $n=5$ per group. Representative images are shown. See the online-only Data Supplement for densitometry.

vehicle-infused mice, high salt intake did not affect pressure. Norepinephrine also increased the abundance of NCC and phosphorylated (activated) NCC (pNCC; Figure 1B and 1C; Figure S1A and S1B in the online-only Data Supplement). These results are similar to those reported previously by others,⁸ although we did not detect changes in the abundance of the NCC regulatory kinase WNK4, at least at the protein level (Figure 1C; Figure S1C).

Although these results confirm that chronic norepinephrine administration increased blood pressure and activated NCC, the effects may not have been direct. To develop an *in vivo* approach that limits compensatory changes, we determined norepinephrine effects 30 minutes after administration. Indeed, pNCC was increased at this time point; in contrast, and as expected, total NCC abundance remained unchanged (Figure 2A and 2B; Figure S2A–S2E). To determine whether norepinephrine activation of NCC requires angiotensin II, a known NCC activator,^{22,23} angiotensin II receptor type 1a knockout mice were treated with norepinephrine. These animals have normal NCC and pNCC abundance at baseline (Figure 2C; Figure S2F and S2G), and the effect on pNCC 30 minutes after norepinephrine treatment was preserved (Figure 2D; Figure S2H and S2I).

To test whether α - or β -receptors were responsible for rapid NCC activation, we used the α_1 -specific agonist phenylephrine and the β_1/β_2 -specific agonist isoproterenol. Stimulation of α_1 -receptors with phenylephrine did not increase pNCC significantly (Figure 3A; Figure S3A and S3B), whereas isoproterenol-mediated stimulation of β -receptors did (Figure 3B; Figure S3C and S3D). Simultaneous stimulation of α_1 - and β -adrenergic receptors with both phenylephrine and isoproterenol increased pNCC more than either agonist alone, suggesting a potential interaction of receptor subtypes (Figure 3C; Figure S3E and S3F).

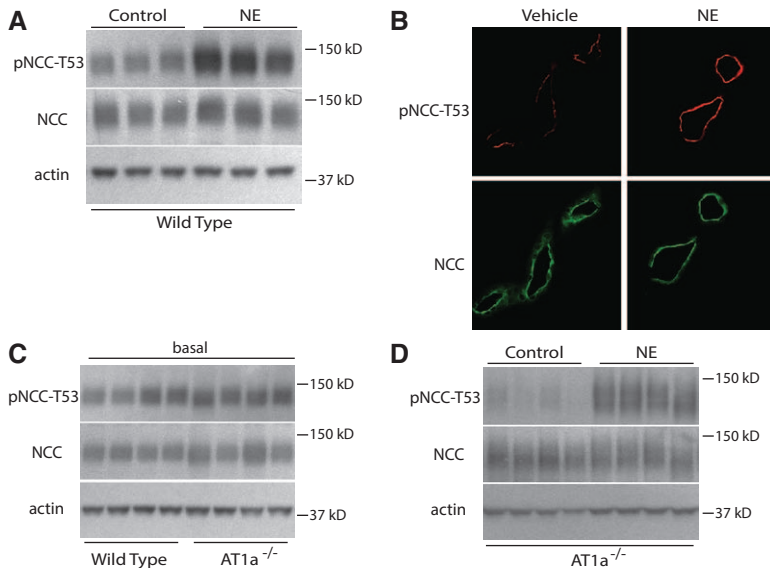


Figure 2. Norepinephrine (NE) rapidly increases phosphorylated NaCl cotransporter (pNCC) independent of angiotensin II signaling. **A**, Treatment with NE for 30 min increased pNCC-T53 abundance, but did not alter total NCC; $n=8$ per group; $P<0.05$ by unpaired t test. **B**, Increased pNCC-T53 abundance could also be observed by immunofluorescence staining. Staining was performed on 3 mice per group. **C**, Genetic deletion of the angiotensin II receptor type 1a (AT_{1a}) does not alter NCC or pNCC-T53 abundance compared with wild-type (WT) controls; $n=7$ per group. **D**, Treatment with NE for 30 min in AT_{1a}^{-/-} mice increased pNCC-T53 compared with control animals; $P<0.05$ by unpaired t test. Total NCC abundance remained unchanged; $n=5$ per group. Representative images are shown. See the online-only Data Supplement for densitometry.

Although a role for adrenergic receptors in modulating renal renin release is well documented, the role and nature of adrenergic receptors on distal nephron cells is not as clear. We first tested whether DCT cells express β -adrenergic receptors by isolating DCT cells from kidney cortex of mice expressing green fluorescent protein only in the DCT using a fluorescent sorting technique, described previously.²⁴ RNA isolated from whole kidney and DCT was then used for reverse transcription polymerase chain reaction. Although both β_1 - and β_2 -receptors were detected (Figure 4A), β_1 -receptors were highly enriched in DCT cells. In contrast, β_2 -receptors were not, consistent with the results of an unbiased genetic screen of the same cells.²⁵ When isolated DCT cells were exposed *ex vivo* to isoproterenol, pNCC increased after 10 minutes (Figure 4B).

Mu et al⁸ suggested that the WNK kinase pathway is involved in chronic adrenergic effects on NCC. WNKs signal NCC predominantly via SPAK, the major serine/threonine kinase that phosphorylates and activates NCC. To test whether adrenergic agonists activate SPAK, we examined the effects of norepinephrine on SPAK abundance and cellular localization. Norepinephrine administration did not affect SPAK localization within DCT cells and did not increase SPAK abundance, at least within 30 minutes (Figure 5A and 5B; Figure S4A). It also did not cause an isoform switch,

which corresponds to SPAK activation.^{26,27} These results, however, do not indicate whether SPAK is necessary for norepinephrine to stimulate NCC. Thus, we tested the effects of norepinephrine in SPAK^{-/-} mice. Although pNCC abundance at baseline is low in these mice,^{26,27} pNCC increased robustly (Figure 5C and 5D; Figure S4B). In fact, the proportional increase in pNCC abundance was larger in SPAK^{-/-} mice than in wild-type mice (Figure S4C).

Because OxsR1 has also been shown to phosphorylate NCC *in vitro*,²⁸ and it is expressed along the DCT, we tested whether norepinephrine alters its cellular localization. In both wild-type and SPAK^{-/-} mice, norepinephrine strikingly altered cellular OxsR1 appearance by immunofluorescence (Figure 6). Treatment with norepinephrine caused a striking increase in apical OxsR1, which assumed a ribbon-like appearance near the apical membrane of the DCT. This was especially obvious in the SPAK^{-/-} mice, because OxsR1 assumes a distinctive punctate pattern in SPAK^{-/-} mice, at baseline^{26,27,29} (Movie S1).

Although these results suggested a role for OxsR1 in mediating the effects of norepinephrine, they do not prove that it is essential. To determine whether OxsR1 is essential for increasing the abundance of pNCC, we generated doxycycline-inducible kidney-specific OxsR1^{-/-} mice. Total-body OxsR1 is embryonic-lethal, so we deleted OxsR1 along the nephron

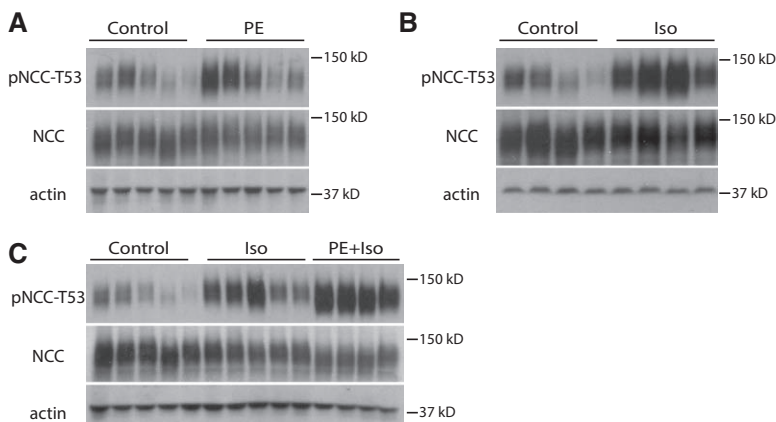


Figure 3. β -Receptors mediate the effects of norepinephrine on NaCl cotransporter (NCC). **A**, Treatment with the α -receptor agonist phenylephrine (PE) for 30 min did not significantly increase pNCC-T53. Total NCC abundance also remained unchanged; $n=5$ per group. **B**, Treatment with the β -receptors agonist isoproterenol (Iso) for 30 min significantly increased pNCC-T53 abundance; $P<0.05$ by unpaired t test. Total NCC abundance remained unchanged; $n=4$ per group. **C**, Treatment with both agonists together increased pNCC-T53 abundance greater than either agonist alone; $P<0.016$ by unpaired t test. Total NCC abundance remained unchanged; $n=5$ for the control and Iso groups and $n=4$ for the Iso plus PE group. See the online-only Data Supplement for densitometry.

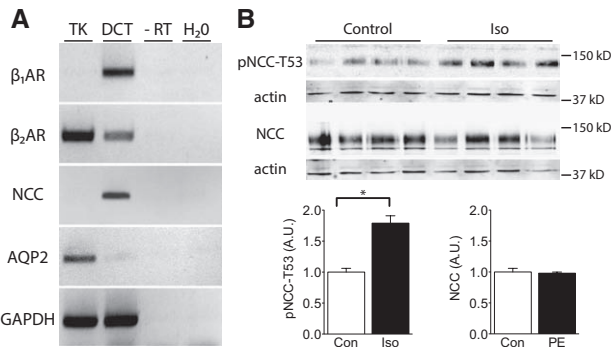


Figure 4. Distal convoluted tubule (DCT)-specific reverse transcription polymerase chain reaction (RT-PCR) for β -adrenergic receptor subtypes. **A**, RT-PCR on fluorescence dissected DCT cells indicated that both β_1 - and β_2 -adrenergic receptors are expressed in the DCT, but the β_1 -receptor subtype is highly enriched. The template used for RT-PCR was either 5 ng complementary DNA (cDNA) produced from RNA extracted from total kidney (TK) or fluorescence dissected DCT cells (DCT). In place of template cDNA, negative control reactions contained either the product from a cDNA reaction using DCT RNA that lacked reverse transcriptase (-RT) or H_2O . β_1 AR indicates β_1 -adrenergic receptor; β_2 AR, β_2 -adrenergic receptor; AQP2, aquaporin-2; Con, control; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; and NCC, NaCl cotransporter. **B**, Ex vivo treatment of fluorescence dissected DCT cells with isoproterenol (Iso) for 10 min increased pNCC-T53 abundance, but did not alter total NCC; $n=4$ per group. A.U. indicates arbitrary units. Graphs depict mean \pm SEM; * $P<0.05$ by unpaired t test.

using the Pax8 LC1 system (Figure 7A; Figure S5A). After deletion of OxSR1, these mice have normal NCC abundance (both total and phosphorylated; Figure 7B; Figure S5B and S5C). Thirty minutes after norepinephrine administration, pNCC abundance increased in these mice, but in contrast to the previously performed experiments, the increase was severely blunted (Figure 7C; Figure S5D and S5E). This suggests that OxSR1 is essential for the full effect of norepinephrine on NCC.

Discussion

The sympathetic nervous system plays a central role in human blood pressure control. Sympathetic nerves to the kidney contribute importantly to these effects by stimulating renal renin

release, modulating renal vascular tone, and activating NaCl transporters.¹ Ablating renal sympathetic nerves is an old approach to resistant hypertension that has recently regained favor, owing to technical advances, which make it easier and safer. In many animal and human studies, this approach has reduced arterial pressure substantially¹²; yet a rigorous and large clinical trial recently could not confirm that renal denervation is superior to standard care. Thus, although renal denervation reduces blood pressure in models of hypertension, its role in clinical medicine remains to be established.

Here, we confirmed that norepinephrine causes salt-sensitive hypertension in mice, in part, by activating salt reabsorption along the distal nephron via NCC. We show that the effects of norepinephrine on NCC activity occur rapidly and are largely mediated via β -adrenergic receptors; we also identify a novel role for OxSR1 in stimulating NCC in response to stress. Although this inquiry has largely focused on the abundance of pNCC, our immunofluorescence data also suggest that norepinephrine may induce a translocation of NCC from the cytosol to the apical membrane (Figure 2B), as reported previously for angiotensin II.²² In our acute studies, we could not measure salt transport directly, but the abundance of pNCC is used widely as a surrogate for NCC activity.^{23,30} Furthermore, Mu et al⁸ reported that thiazide treatment reverses norepinephrine-induced hypertension, supporting a functional role for NCC activity in these effects. The finding that reducing salt intake lowers pressure during continued norepinephrine infusion⁸ further supports the importance of salt and is consistent with the effects of salt loading in several other experimental models.³¹ The current results clarify the important role that NaCl reabsorption plays in the effects of norepinephrine on arterial pressure and suggest one reason that SYMPPLICITY HTN-3 may have failed to achieve its primary goal.

The nature of the direct and indirect effects of adrenergic stimulation on the kidney has been debated for many decades. Here, we show that the effect of norepinephrine to increase pNCC rapidly is fully preserved in mice lacking angiotensin II receptor type 1a, indicating that the effect is not mediated by renin or angiotensin II. The effect seems to be mediated instead through β -adrenergic receptors, as observed previously

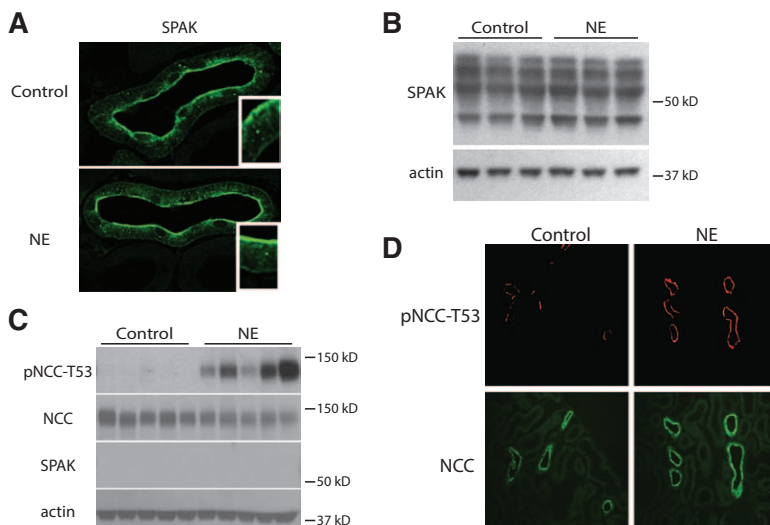


Figure 5. STE20/SPS1-related proline- and alanine-rich kinase (SPAK) is not essential for the rapid effects of norepinephrine (NE) on NaCl cotransporter (NCC). **A**, Treatment with NE for 30 min did not alter SPAK cellular localization within the DCT in wild-type animals. Staining was performed on 3 mice per group. **B**, Treatment with NE for 30 min did not alter SPAK abundance in wild-type animals; $n=3$ per group. **C**, Treatment with NE for 30 min in SPAK^{-/-} animals significantly increased pNCC-T53 abundance; $P<0.05$ by unpaired t test. Total NCC protein remained unchanged; $n=5$ per group. **D**, Increased pNCC-T53 abundance in SPAK^{-/-} animals could also be observed by immunofluorescence staining. Staining was performed on 3 mice per group. See the online-only Data Supplement for densitometry.

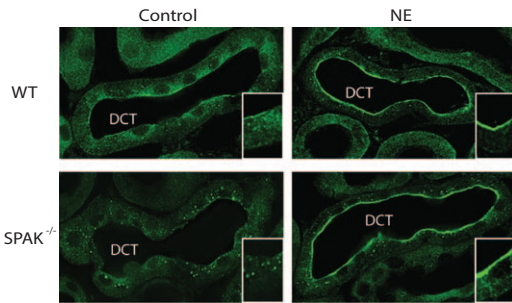


Figure 6. Norepinephrine (NE) increased distal convoluted tubule (DCT) oxidative stress–response kinase 1 (OxsR1) apical localization in both wild-type (WT) and STE20/SPS1-related proline- and alanine-rich kinase (SPAK)–deficient (SPAK^{-/-}) mice. Treatment with NE for 30 min increased the apical localization of OxsR1 in the DCT of both WT and SPAK^{-/-} mice. Insets are high-magnification images of the represented tubule sections. Staining was performed on 3 mice per group.

during chronic treatment.⁸ Yet, we also detected evidence for synergistic interactions between α - and β -adrenergic receptors to stimulate NCC, a finding compatible with previously contradictory results. Although we find that both β_1 - and β_2 -adrenergic receptor subtypes are expressed in the DCT, β_1 -receptors were highly enriched, suggesting that they play a dominant role. This is consistent with anatomic studies performed in the 1970s and 1980s by Barajas et al.^{32,33} Taken together, our results suggest that NCC can be modulated rapidly by direct β -adrenergic input to DCT cells, probably enhanced by concomitant α -adrenergic stimulation.

Although many groups have observed that norepinephrine infusion increases blood pressure, especially in the setting of dietary NaCl loading,^{7,8} the effects of this treatment on NCC

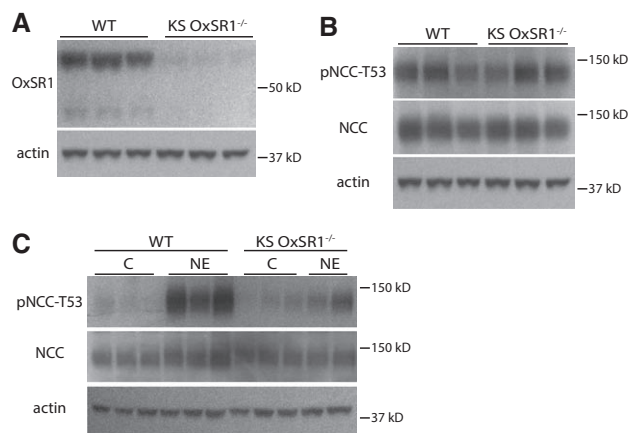


Figure 7. Kidney-specific (KS) oxidative stress–response kinase 1 (OxsR1)–deficient (OxsR1^{-/-}) mice exhibited a diminished response to norepinephrine (NE). **A**, KS OxsR1^{-/-} mice had significantly decreased abundance of OxsR1 in their kidneys compared with wild-type (WT) controls; $n=3$ per group; $P<0.05$ by unpaired t test. **B**, Abundance of NaCl cotransporter (NCC) protein and phosphorylation at threonine-53 was unaltered in KS OxsR1^{-/-} mice at baseline; $n=3$ per group. **C**, KS OxsR1^{-/-} mice exhibited a blunted response to a 30-min treatment with NE. NCC phosphorylation at threonine-53 increased in these mice, but to a lesser degree than that observed in wild-type controls; $P<0.05$ by unpaired t test; $n=3$ for WT per group mice and 4 for KS OxsR1^{-/-} mice per group. **C**, Control (C) representative images are shown. See the online-only Data Supplement for densitometry.

and pNCC abundance, as well as WNK signaling cascade activity, have been more controversial. Our data here confirm that norepinephrine infusion chronically increases both NCC and pNCC and raises arterial pressure, as shown previously by Mu et al.⁸ Uchida et al⁷ confirmed the blood pressure effects of norepinephrine infusion with dietary salt loading, but could not detect the effects on NCC and WNK4. Although the reasons for these discrepancies are not clear, acute hypertension or dietary salt loading typically downregulates NCC^{34,35}; thus, the absence of this effect during norepinephrine infusion noted by Uchida et al⁷ likely reflects abnormal NCC activation.

NCC and the furosemide-sensitive Na-K-2Cl cotransporter are stimulated by a signaling cascade that includes WNK kinases, SPAK and OxsR1. We found no change in WNK4 abundance on norepinephrine stimulation, using an antibody validated against WNK4-knockout tissue, suggesting that WNK4 is not involved. Similarly, we found no apparent effect of norepinephrine on SPAK. SPAK deletion strikingly reduces the abundance of pNCC in vivo,^{26,27,36,37} whereas OxsR1 deletion does not.³⁸ This has led to a simple paradigm in which SPAK, and not OxsR1, was viewed as responsible for activating NCC.^{26,38} Surprisingly, adrenergic stimulation induced a shift in OxsR1 toward the apical membrane of DCT cells, where it might be expected to phosphorylate and activate NCC (Figure 8). This apical redistribution occurs whether or not SPAK is present, suggesting that this does not require SPAK/OxsR1 interactions. Because OxsR1 is capable of phosphorylating cation chloride cotransporters in vitro,³⁹ this suggests that OxsR1 plays an essential and nonredundant role in the adrenergic response. In confirmation of this, inducing OxsR1 deletion along the nephron, which did not affect basal pNCC abundance, strikingly blunted the ability of norepinephrine to increase pNCC.

A role for OxsR1 in regulating NCC was suggested by Chiga et al,⁴⁰ who reported that OxsR1 kinase activity is required for the full familial hyperkalemic hypertension phenotype. They found that disrupting SPAK kinase activity did

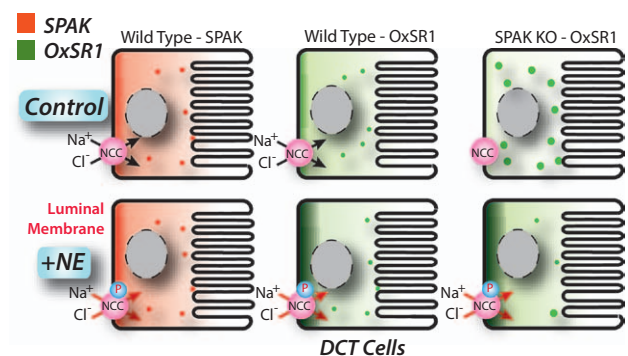


Figure 8. Model of norepinephrine (NE) effects on STE20/SPS1-related proline- and alanine-rich kinase (SPAK) and oxidative stress–response kinase 1 (OxsR1) in the distal convoluted tubule. Cartoon shows effects of NE on SPAK (red) and OxsR1 (green) distribution in wild-type mice (**left two panels**). Note that OxsR1, but not SPAK, shifts to an apical location. The right panels show effects of NE on OxsR1 in SPAK-knockout (KO) mice. Notice that SPAK KO causes OxsR1 to shift to a more punctate appearance. On NE stimulation, OxsR1 shifts substantially toward the apical membrane. NaCl cotransporter (NCC) is phosphorylated (P) on activation. DCT indicates distal convoluted tubule.

not completely ameliorate the familial hyperkalemic hypertension phenotype. Instead, the disruption of both OxsR1 and SPAK was necessary to return NCC phosphorylation, blood pressure, and plasma K^+ back to normal. Grimm et al²⁷ postulated that OxsR1 requires SPAK to move to the apical membrane, where it can phosphorylate NCC. The current results indicate that this deficiency can be overcome by adrenergic stimulation, because apical OxsR1 is abundant after stimulation in SPAK^{-/-} mice (Figure 8). Taken together, the results suggest complementary, but distinct, roles for SPAK and OxsR1 in regulating NCC. Clearly, SPAK is the dominant NCC-activating kinase under basal conditions, but OxsR1 seems to predominate when the kidney responds to adrenergic stimulation. As suggested by Grimm et al,²⁷ both kinases may be necessary to activate NCC to its fullest extent.

Guyton⁴¹ suggested that, although the kidney is an especially potent regulator of arterial pressure, these actions are slow. It has been surprising, therefore, to find that NCC is modulated acutely in a variety of circumstances. Acute hypertension, or angiotensin-converting enzyme inhibition, shifts NCC out of the plasma membrane^{22,34,42}; acute KCl gavage rapidly reduces pNCC abundance.⁴³ Here, we have shown that adrenergic stimulation activates NCC rapidly and substantially. This response is most likely a part of the fight-or-flight reflex, described by Cannon,⁴⁴ in which the maintenance of arterial pressure and essential organ perfusion provides short-term advantage. When this response is anomalously derived and maintained chronically, however, it likely contributes to pathological hypertension. There has been substantial disappointment that renal denervation did not prove effective in the large rigorous SYMPPLICITY HTN-3 trial for the treatment of resistant hypertension.¹⁴ Some commentators have suggested that one difference between the SYMPPLICITY HTN-3 trial and earlier trials is the intense focus on appropriate medical management in the SYMPPLICITY HTN-3.⁴⁵ This focus included aggressive use of thiazide-type diuretics and aldosterone antagonists, both which inhibit NCC.⁴⁶ Thus, these drugs may have achieved, medically, some of the effects that nerve ablation would otherwise, rendering the intervention less effective.

Perspectives

The current work shows that adrenergic stimulation of β -receptors in DCT cells stimulates NCC, both acutely and chronically, contributing to salt-sensitive hypertension. This effect is mediated largely by OxsR1, rather than SPAK, identifying an essential and nonredundant role for OxsR1 in modulating NCC activity, a role that was previously unappreciated. The results suggest that renal nerves exert important effects on the kidney by activating salt transport pathways to increase arterial pressure; for this reason, the benefits of invasive procedures may not seem as great, when compared with a control group treated aggressively with drugs that block salt transport.

Acknowledgments

A.S. Terker and D.H. Ellison are grateful to Virginia Brooks, PhD, for advice and support in the completion of this work. We wish to acknowledge Aurelie Snyder and the Advanced Light Microscopy Core at The Junegers Center, OHSU, for technical assistance.

Sources of Funding

This work was performed in partial fulfillment of the requirements for a PhD by A.S. Terker. It was supported by a Predoctoral Fellowship from the American Heart Association (13PRE14090030) and by grants from the National Institutes of Health (NIH) and Department of Veterans Affairs to D.H. Ellison (DK51496 and Merit Review) and from NIH to E. Delpire (GM074771 and DK093501). It was also supported by Shared Instrumentation Grant Number S10-RR023432 from the National Center for Research Resources (NCRR), a component of the NIH. Its contents are solely the responsibility of the authors and do not necessarily represent the official views of NCRR or NIH.

Disclosures

None.

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Novelty and Significance

What Is New?

- Adrenergic stimulation rapidly activates NaCl cotransporter in the distal tubule via β -adrenergic receptors, which are enriched in distal convoluted tubule cells. The effect is not dependent on angiotensin II or STE20p-related proline- and alanine-rich kinase and is largely mediated by the oxidative stress-response kinase 1. These effects seem to contribute importantly to salt-sensitive hypertension resulting from norepinephrine infusion.

What Is Relevant?

- These findings help explain how excessive sympathetic nerve activity causes hypertension. They indicate that medical and device-based approaches to hypertension converge on common mechanisms.

Summary

Our data provide evidence for an in vivo β -adrenergic signaling cascade in the distal convoluted tubule. It is largely mediated by the oxidative stress-response kinase 1 and is relevant in the generation of salt-sensitive hypertension.

**SYMPATHETIC STIMULATION OF THIAZIDE-SENSITIVE SODIUM-
CHLORIDE COTransport IN THE GENERATION OF SALT-SENSITIVE
HYPERTENSION
Online Supplement**

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Detailed Methods

Animals

All animal studies were approved by Oregon Health and Science University's Institutional Animal Care and Usage Committee (Protocol IS918). All mice were male littermates (12–16 weeks, 25–30 g) and had a C57Bl/6 background. All strains are backcrossed to the appropriate wild-type mice every 10 generations to maintain genetic backgrounds. SPAK^{-/-} mice,^{21,22} and Angiotensin II receptor type 1a (AT_{1a})^{-/-} mice²³ have all been reported previously. Doxycycline-inducible kidney-specific (KS) OxSR1^{-/-} mice were generated by crossing mice that were homozygous for floxed OxSR1 with *loxP* sites flanking the 113 bp exon 2 with mice that express both the reverse tetracycline transactivator (rtTA) under the control of the renal epithelial cell-specific Pax8 promoter²⁴ and the LC1 transgene.²⁵ Wild-type controls for the KS OxSR1^{-/-} mice were those littermates that had two floxed OxSR1 alleles, but lacked either the LC1 transgene, the Pax8 transgene, or both. Prior to use in reported studies, KS OxSR1^{-/-} mice were treated with doxycycline (2 mg/ml in 5% sucrose in drinking water) for 15 days to induce Cre-mediated recombination. Wild-type controls also received doxycycline.

PCR Genotyping

Standard PCR on tail snips was used for animal genotyping. For AT_{1a}^{-/-} mice, the following primers were used to distinguish knockout from wild-type animals: 5'-TGAGAACACCAATATCACTG-3', 5'-TTCGTAGACAGGCTTGAG-3', and 5'-CCTTCTATCGCCTTCTTGACG-3'. To distinguish mice carrying at least one modified SPAK allele from wild-type mice, a previously reported approach was used.²² Western blots on protein extracts from cultured tail cells were then used to further distinguish heterozygous SPAK mice from homozygous SPAK null mice. For KS OxSR1^{-/-} mice, the following primers were used to identify animals that were homozygous for floxed exon 2 within the OxSR1 gene and also carried at least one copy each of the Cre and Pax8 rtTA transgenes: OxSR1FloxF: 5'-AGCTCAGGCTCCCTCCACGGAG, OxSR1FloxR: 5'-AAGACACATTGATACTCTGTTTCTCGAAGG; CreF: 5'-TTTCCCGCAGAACCTGAAGATG, CreR: 5'-TCACCGGCATCAACGTTTTCTT; Pax8F: 5'-CCATGTCTAGACTGGACAAGA, Pax8R: 5'-CAGAAAGTCTTGCCATGACT.

Animal studies

Systolic blood pressures (SBPs) were measured with tail-cuffs using volumetric pressure recording (CODA-6; Kent Scientific). This method has been validated for SBP measurements in mice, showing correlation with radiotelemetry.^{26,27} The mice were first acclimated to the experimental procedure for five consecutive days; baseline SBP was then measured for one week on normal salt (0.49% NaCl). At the start of the second week, NE (2.5 mg/kg/d, Sigma) or vehicle was administered via osmotic minipump (Alzet) for two weeks while SBP measurements were continued. During the third week, all mice were switched to a high salt diet (8% NaCl, Harlan Teklad) and

SBP measurements were continued. For short-term adrenergic stimulation studies, norepinephrine (1250 ug/kg), phenylephrine (1250 ug/kg), isoproterenol (1250 ug/kg), or vehicle was injected intraperitoneally as indicated.

Immunoblotting

Mouse kidneys were harvested and snap-frozen in liquid nitrogen. Kidneys were then homogenized on ice in chilled homogenization buffer containing protease and phosphatase inhibitors. Protein (20–80 µg) was separated on 3–8% (wt/vol) Tris acetate or 4–12% (wt/vol) Bis-Tris gel (Invitrogen). All primary antibodies have been characterized and were specific to the following proteins: NCC, pNCC at threonine-53, and WNK4 (developed in laboratory of DHE), pNCC at threonine-58 and pNCC at serine-71 (developed in laboratory of JL), SPAK, OxSR1,²⁸ β-actin (Abcam). Densitometry was performed using ImageJ (<http://rsbweb.nih.gov/ij/>). All bands were normalized to actin. Representative images are shown. Densitometry is in the online supplement.

Immunofluorescence

Mice were anesthetized with isoflurane and kidneys perfusion-fixed by retrograde abdominal aortic perfusion of 3% paraformaldehyde in PBS (pH 7.4). After overnight cryoprotection with 800 mOsm sucrose and freezing, 5 µm sections were cut, washed in 1× PBS, incubated in 1× PBS with 0.5% Triton X for 30 min, washed in PBS, and blocked in 5% milk in PBS for 30 min. Primary antibody in 5% milk in 1× PBS was added for 1 hr, followed by a wash in PBS. Sections were incubated for 45 min with secondary antibody in block, then washed. Images were acquired on a high resolution wide field Core DV system (Applied Precision™). The system was an Olympus IX71 inverted microscope with a proprietary XYZ stage enclosed in a controlled environment chamber; differential interference contrast (DIC) transmitted light and a solid state module for fluorescence. The camera used was a Nikon Coolsnap ES2 HQ. Representative images are shown.

DCT-Specific PCR

DCT cDNA was obtained from mice expressing the enhanced green fluorescent protein (EGFP) under the control of the parvalbumin promoter. GFP+ tubule segments were isolated via a Complex Object Parametric Analyzer and Sorter (COPAS) and RNA and cDNA were prepared all as described previously.²⁹ The following primers were used for the designated RT-PCR reactions. β₁-adrenergic receptor forward: 5'-ATCGTTCTGCTCATCGTGGTGGGTAACG, β₁-adrenergic receptor reverse: 5'-CGTCAGCAAACCTCTGGTAGCGAAAGGG; β₂-adrenergic receptor forward: 5'-ATGGGGCCACACGGGAACGAC, β₂-adrenergic receptor reverse: 5'-GGCGTAGGCCTGGTTCTGTAAGAAGTC; NCC forward: 5'-TCACCATCAGCACACTGATGGGAGG, NCC reverse: 5'-ATCACTCCCCAGATGTTGAGCATGCAAC; AQP2 forward: 5'-

GAGATTACCCCTGTAGAAATCCGCGGGG, AQP2 reverse: 5'-GGGTCCGATCCAGAAGACCCAGTGATC; GAPDH forward: 5'-ATGGTGAAGGTCGGTGTGAACGGATTTG, GAPDH reverse: 5'-GCCTTCTCCATGGTGGTGAAGACACCAG.

***Ex vivo* stimulation of COPAS-sorted DCT with Isoproterenol**

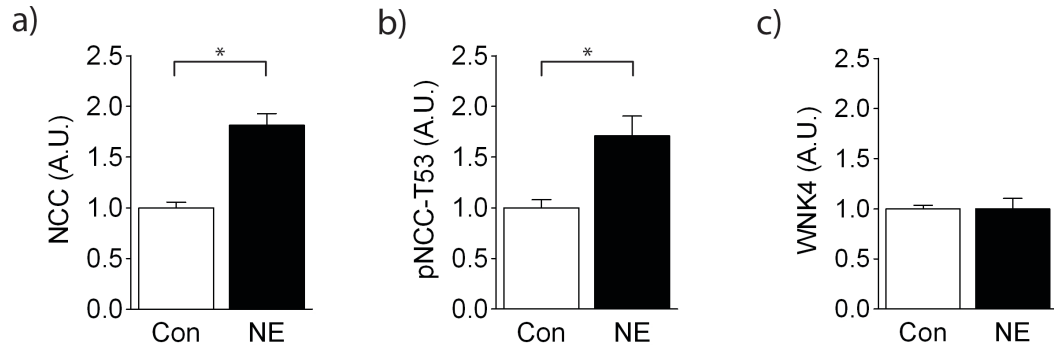
DCTs were sorted with COPAS from one mouse expressing the enhanced green fluorescent protein (EGFP) under the control of the parvalbumin promoter as described previously²⁹. Per condition, 1000 DCTs were sorted into 35 mm Petri dishes (on ice) in an alternating manner (control, isoproterenol, control, isoproterenol, etc.) to avoid time-dependent sorting effects. Once 1000 DCTs per condition were sorted, Petri dishes were placed in an incubator for 5 min until 37°C were reached. Cells were then stimulated with either vehicle or 100 nM isoproterenol (DL-isoproterenol hydrochloride, Sigma) at 37°C for 10 min. The supernatant was collected and the remaining cells washed off the petri dish with ice cold KREBS and collected as well. Cells were spun at 800xg, 4°C for 5 min and the pellet re-dissolved in 30 ul 1x Laemmli. 500 tubules were loaded on a 10 % SDS PAGE to detect either NCC or pNCC.

Statistical Analyses

Unpaired t-tests were used to compare groups. $p < 0.05$ was considered significant. When necessary, corrections for multiple comparisons were used as indicated. Two-way ANOVA with repeated measures was used to compare blood pressure across time in vehicle- and norepinephrine-treated mice.

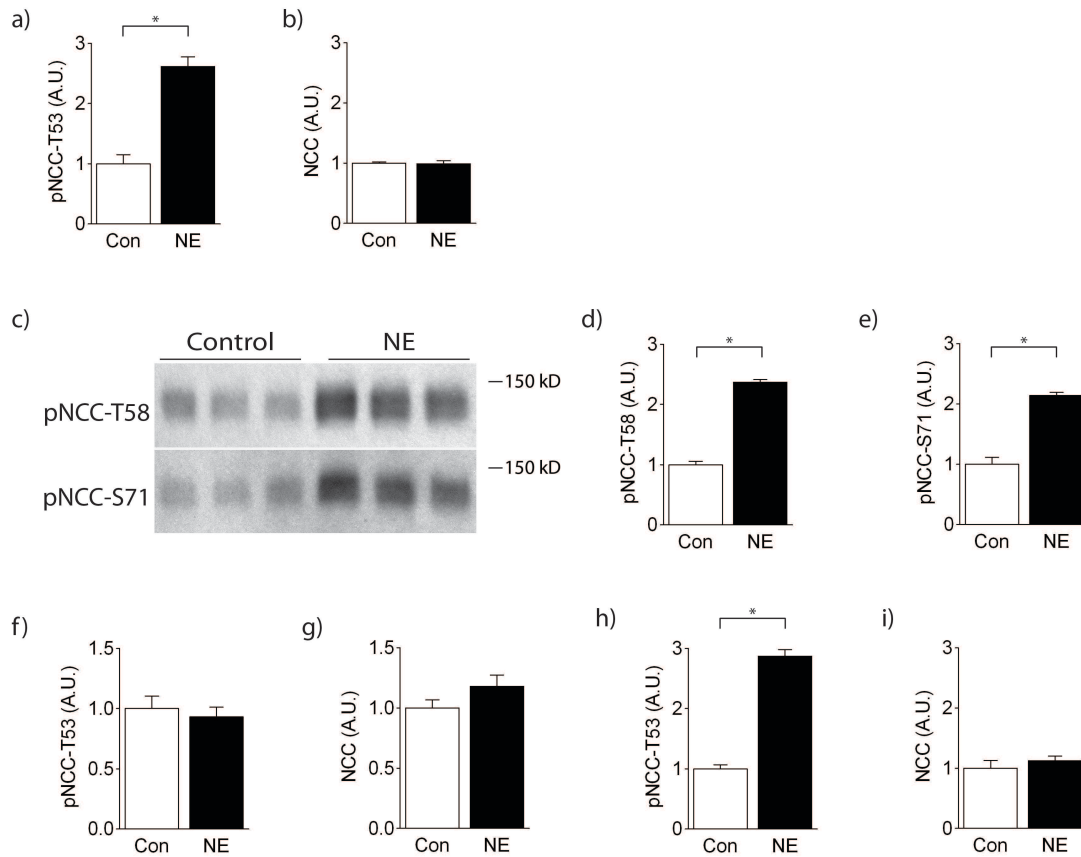
Supplemental Figure Legends

S1



S1: Western blot quantification for chronic NE treatment. Panel A:

Chronic NE treatment increased NCC abundance. **Panel B:** Chronic NE treatment increased pNCC-T53 abundance. **Panel C:** WNK4 abundance was unchanged by chronic NE treatment. Graphs depict mean \pm s.e.m. * p < 0.05 by unpaired t-test.



S2: Western blot quantification for acute NE treatment. Panel A:

Treatment with NE for 30 minutes increased pNCC-T53 abundance. **Panel B:**

Treatment with NE for 30 minutes did not change NCC abundance. **Panel C:**

Treatment with NE for 30 minutes also increased pNCC-T58 and pNCC-S71

abundance. **Panel D:** Immunoblot quantification for pNCC-T58 abundance.

Panel E: Immunoblot quantification for pNCC-S71 abundance. **Panel F:** pNCC

abundance in $AT_{1a}^{-/-}$ mice is not statistically different from wild-type

controls. **Panel G:** NCC abundance in $AT_{1a}^{-/-}$ mice is not statistically different

from wild-type controls. **Panel H:** Treatment with NE for 30 minutes

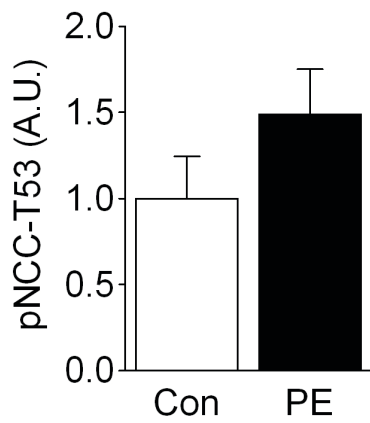
increased pNCC-T53 abundance in $AT_{1a}^{-/-}$ mice. **Panel I:** Treatment with NE

for 30 minutes did not change NCC abundance in $AT_{1a}^{-/-}$ mice. Graphs depict

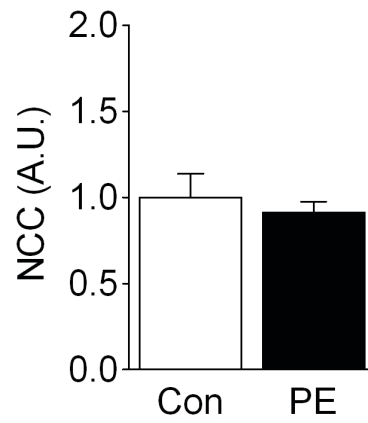
mean \pm s.e.m. * $p < 0.05$ by unpaired t-test.

S3

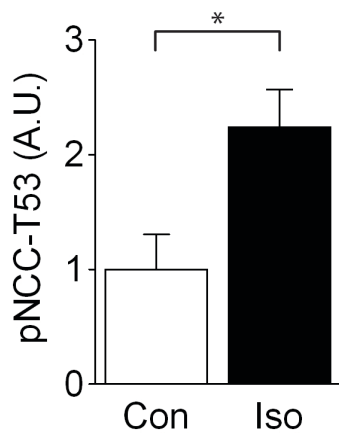
a)



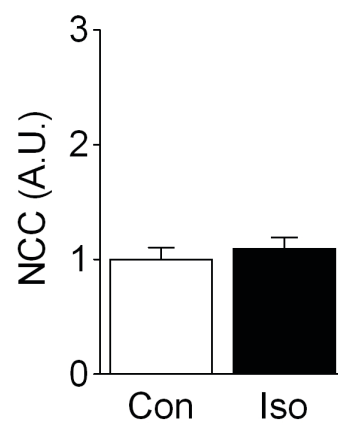
b)



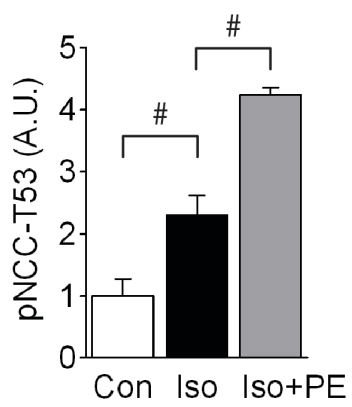
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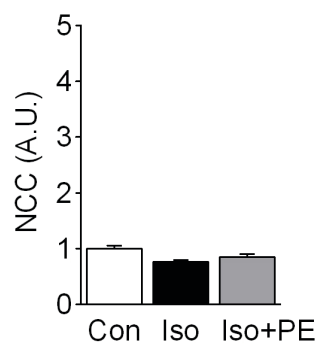
d)



e)

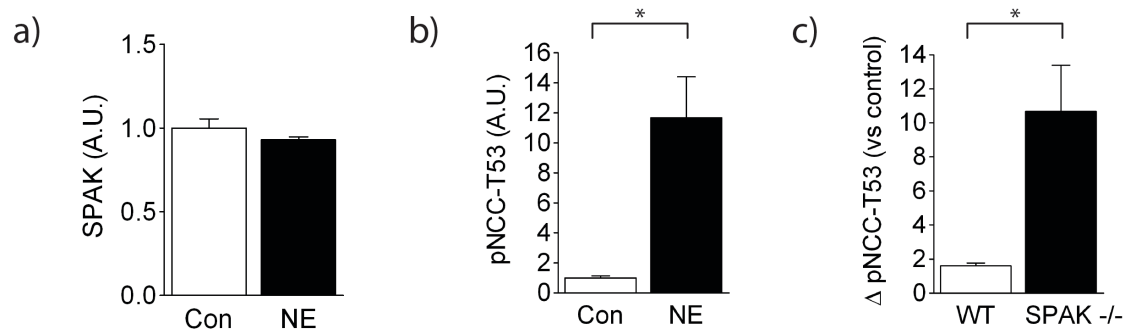


f)



S3: Western blot quantification for acute PE and Iso treatment. Panel A: Treatment with the α -receptor agonist, phenylephrine (PE), for 30 minutes did not significantly increase pNCC-T53 abundance. **Panel B:** Treatment with PE for 30 minutes did not change NCC abundance. **Panel C:** Treatment with the β -receptors agonist, isoproterenol (Iso), for 30 minutes significantly increased pNCC-T53 abundance. **Panel D:** Treatment with Iso for 30 minutes did not change NCC abundance. **Panel E:** Treatment with both agonists together increased pNCC-T53 abundance greater than either agonist alone. **Panel F:** Treatment with both agonists together did not alter NCC abundance. Graphs depict mean \pm s.e.m. * $p < 0.05$ by unpaired t-test. # $p < 0.016$ by unpaired t-test.

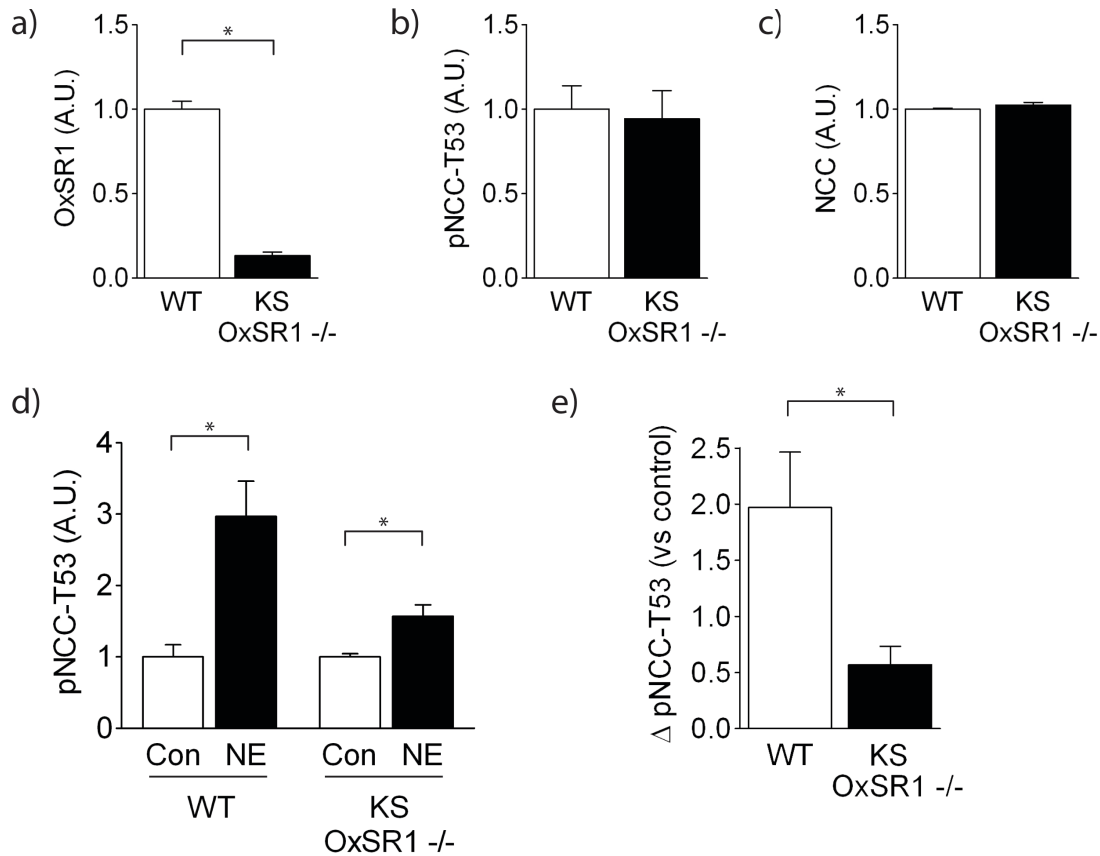
S4



S4: Western blot quantification for acute NE treatment in SPAK^{-/-} mice.

Panel A: Treatment with NE for 30 minutes did not change SPAK abundance in wild-type mice. **Panel B:** Treatment with NE for 30 minutes increased pNCC-T53 abundance in SPAK^{-/-} mice. **Panel C:** Treatment with NE for 30 minutes increased pNCC-T53 abundance in SPAK^{-/-} mice greater than in wild-type controls. Graphs depict mean ± s.e.m. * p<0.05 by unpaired t-test.

S5



S5: Western blot quantification for acute NE treatment in KS OxSR1^{-/-} mice. Panel A: KS OxSR1^{-/-} mice had significantly decreased abundance of OxSR1 in their kidneys compared with wild-type controls. **Panel B:** The abundance of pNCC-T53 was unaltered in KS OxSR1^{-/-} mice at baseline. **Panel C:** The abundance of NCC protein was unaltered in KS OxSR1^{-/-} mice at baseline. **Panel D:** Treatment with NE for 30 minutes increased pNCC-T53 abundance in KS OxSR1^{-/-} mice. **Panel E:** Treatment with NE for 30 minutes increased pNCC-T53 abundance in KS OxSR1^{-/-} mice less than in wild-type controls. Graphs depict mean \pm s.e.m. * $p < 0.05$ by unpaired t-test.

Supplemental Movie 1: NE increased DCT OxsR1 apical localization in SPAK^{-/-} mice. Treatment with NE for 30 minutes increased the apical localization of OxsR1 in the DCT of SPAK^{-/-} mice.